DOI 10.1002/etc.157

ENVIRONMENTAL OCCURRENCE AND REPRODUCTIVE EFFECTS OF THE PHARMACEUTICAL FLUOXETINE IN NATIVE FRESHWATER MUSSELS

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Running Head: Fluoxetine effects on freshwater mussels

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Additional Supporting information may be found in the online version of this article.

Submitted 11 August 2009; Returned for Revision 9 December 2009; Accepted 9 January 2010

Abstract− The present study measured the occurrence, distribution, and bioaccumulation of fluoxetine in samples of water, polar organic chemical integrative sampler (POCIS), sediment, and caged freshwater mussels at stream sites near a municipal wastewater treatment facility effluent discharge. We assessed the relation of the environmental concentrations to reproductive endpoints in mussels in acute laboratory tests. Concentrations of fluoxetine in water and POCIS samples were similar (<20% difference) within each site and were greatest in the effluent channel (104-119 ng/L), and decreased at 50 m and 100 m downstream. Likewise, concentrations of fluoxetine in sediment and mussel (*Elliptio complanata*) tissue were greatest in the effluent channel (17.4 ng/g wet wt for sediment and 79.1 ng/g wet wt for mussels). In 96-h lab tests, fluoxetine significantly induced parturition of nonviable larvae from female *E. complanata* exposed to 300 µg/L ($p = 0.0118$) and 3000 µg/L ($p < 0.0001$) compared to controls. Fluoxetine exposure at 300 µg/L ($p = 0.0075$) and 3000 µg/L ($p = 0.0001$) also resulted in stimulation of lure display behavior in female *Lampsilis fasciola* and *Lampsilis cardium*, respectively. In male *E. complanata*, 3000 µg fluoxetine/L significantly induced release of spermatozeugmata during a 48-h exposure. These results suggest that fluoxetine accumulates in mussel tissue and has the potential to disrupt several aspects of reproduction in freshwater mussels, a faunal group recognized as one of the most imperiled in the world. Despite the disparity between measured environmental concentrations of fluoxetine and effects concentrations in our short-term tests with these long lived animals, additional tests are warranted to evaluate the effects of long term exposure to environmentally-relevant concentrations and critical lifestages (e.g., juveniles).

Keywords− Unionidae, Pharmaceutical, Glochidia, Behavior, Prozac

INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) have been detected in surface waters, effluent from municipal wastewater treatment facilities, seawater, and groundwater [1-3]. Although these compounds are generally not as persistent as traditional priority pollutants (e.g., polychlorinated biphenyls, organochlorine pesticides), the continuous release of PPCPs into rivers and streams from municipal wastewater treatment discharges presents similar exposure conditions as that of a persistent organic pollutant. Many of these compounds enter the environment in their parent form (un-metabolized) or as a mixture of metabolites [1-4]. In addition, some PPCPs have been found to accumulate in fish in effluent-dominated streams [5]. Therefore, compounds that were manufactured with the intent of being bioactive are being discharged into surface and ground waters and may be responsible for adverse effects in aquatic organisms, including endocrine disruption [6,7].

One of the PPCPs detected frequently in surface waters is fluoxetine, the active ingredient in Prozac® (Eli Lilly and Company) [2,3,8]. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and exerts its action by reducing the clearance rate of serotonin (5-hydroxytrypamine), a neurotransmitter, in the synapse following nerve signal transmission. Serotonin is a key mediator for a wide variety of physiological functions in mollusks; serotonin regulates reproductive processes including oocyte maturation [9,10], spawning [10,11], and parturition (release of glochidia) [12]. Serotonin, fluoxetine, and other SSRIs have been used to artificially induce spawning in freshwater and marine bivalves for aquaculture purposes [13,14] and have been investigated as a potential chemical control mechanism (i.e., disruptor of reproduction) for exotic bivalve species like the zebra mussel *Dreissena polymorpha* [9,15].

Native freshwater mussels (family Unionidae) are long-lived (30 -100 years) suspension- and deposit-feeding benthic organisms that live burrowed in sediments of streams and rivers. They are among the most rapidly declining faunal groups in North America; approximately 70% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or already extint [16,17]. The decline of mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to a wide array of human activities that cause pollution and water-quality degradation and habitat alteration and destruction [18,19]. Principally, the stressors associated with human development and urbanization have hastened these population declines over the past 20 to 50 years. In light of the increasing number of surface waters with measurable fluoxetine (and other SSRIs) concentrations and the imperiled status of the freshwater mussel fauna, determination of the effects of environmental exposures of fluoxetine to native freshwater mussels is of utmost importance.

Most Unionids have a unique life history that includes an obligate parasitic larval stage (glochidia). Gravid female mussels release glochidia that must attach to the gills or fins of a suitable host fish for a period of days to weeks for successful transformation to the free-living juvenile stage [20]. Two of the major subfamilies of North American Unionid mussels use distinct strategies to successfully attach to host fish. Mussels of the tribe Lampsilini use elaborate and highly specialized mantle tissue displays (lures) that resemble prey items such as minnows or insect larvae to attract potential host fish [21,22]. As the fish strikes the mantle flap *lure*, hundreds to thousands of glochidia are released into the buccal cavity of the fish where they attach to gills and undergo transformation into juvenile mussels before releasing from the fish and settling into the sediment to become free-living adults. Tribe Amblemini mussels generally release glochidia into the water column in a broadcast strategy whereby their glochidia drift and

encounter host fish by happenstance or via mucous nets. Because of their unique and complex life history, unionid mussels may be among the groups of aquatic organisms most susceptible to PCPPs and other endocrine disrupting compounds (EDCs) that are now found in our surface waters.

The objective of the present study was to measure the occurrence, distribution and bioaccumulation of fluoxetine in samples of water, polar organic chemical integrative sampler (POCIS), sediment, and caged mussels at stream sites upstream, in, and downstream from a municipal wastewater treatment facility. We also assessed the relation of these measured environmental concentrations to reproductive endpoints (release of gametes, parturition of glochidia, mantle lure display behavior) in mussels in a series of acute (≤96-h) laboratory toxicity tests.

MATERIALS AND METHODS

Environmental sampling and analysis

Crabtree Creek, a tributary to the Neuse River, near the Town of Cary (NC, USA) (population of 127,640), was selected for study. Based on streamflow data from the U.S. Geological Survey this stream has a long-term median flow of approximately 56 mil L/d (23 cfs). The Town operates a wastewater treatment facility that uses tertiary treatment with ultraviolet disinfection and has a permitted discharge of 45.42 mil L/d of treated effluent into Crabtree Creek. According to the USGS [23] the 7-d, consecutive low flow with a ten year return frequency (7Q10) for this creek is approximately 4.9 mil L/d (2.0 cfs). Four sampling sites included one reference site on Crabtree Creek located 100 m upstream of the confluence with

wastewater effluent channel; one site at the midpoint of the 50 m long effluent channel; and two sites downstream (50 m and 100 m, respectively) of the confluence with the effluent channel in the mixing zone. At each site, duplicate 4 L grab surface water samples, triplicate POCIS devices, and single composite surficial (top 5 cm) sediment samples (n = 4 grabs-6 grabs each with a stainless steel scoop) were taken on three separate occasions (August, September, and November of 2007). The POCIS samplers (EST) were deployed for 14 d to 21 d intervals during each sampling event. During the final sampling in November, adult *Elliptio complanata* mussels were collected from a relatively uncontaminated, rural, forested section of the Eno River near Hillsborough (NC, USA), with no permitted discharges upstream in the watershed (http://h2o.enr.state.nc.us/basinwide/Neuse/2008/NeuseRiverBasinPlanDRAFT.htm). The mussels were deployed in cages alongside the POCIS devices at each site. Adult *E. complanata* used for bioaccumulation studies in and near the municipal wastewater effluent were not in reproductive condition (i.e., gravid) at the time of the study therefore gender was not able to be determined. Three mussels were held in a cylindrical porous plastic mesh (10 mm openings) cage at each site. Each cage contained 10 cm of surficial sediment from the site to provide cage stability and burrowing substrate for the mussels, which were deployed for 14 d. Water samples were transported on ice to the laboratory and extracted within 2 h of collection. Sediment, mussel and POCIS samples were transported on ice, promptly frozen upon return to the laboratory, and held at -20 °C until extraction and analysis. All samples were analyzed for fluoxetine as described below.

Fluoxetine and fluoxetine-d_6 were purchased as certified analytical standards from Cerilliant; ^{13}C-phenacetin was purchased from Cambridge Isotope Laboratories. Stock solutions of each analyte were prepared in methanol and working solutions were prepared from the stocks.

Fluoxetine-d_6 and ^{13}C-phenacetin were used as the internal standards. All other chemicals were reagent grade or better, obtained from commercial suppliers and used as received. Ultrapure deionized water was obtained with a Milli-Q system.

During extraction, water samples were filtered (0.45 μm, Whatman), acidified with formic acid to a pH of 2.7, spiked with ^{13}C-phenacetin, and solid phase extracted using hexane, methanol, and water conditioned 47 mm C18 Empore™ disks (Varian). The disk was then dried under vacuum and shaker extracted using 20 ml of methanol. The extraction was repeated for a total of 60 ml methanol. The methanol was filtered with UniPrep™ (Whatman), dried using nitrogen evaporation, and reconstituted in 1ml aqueous mobile phase (10 mM ammonium formate in water). Samples were transferred to autosampler vials for analysis.

Sediment samples were homogenized and aliquoted (23 g-29 g wet wt) into 50 ml glass centrifuge tubes for extraction. Samples were spiked with fluoxetine-d_6 and serially extracted three times on a flat bed shaker with 25 ml of a 50:50 mixture of methanol:dichloromethane (volume fraction) and 0.25 ml of ammonium hydroxide solution for 30 min. Samples were centrifuged (1700 rpm; 15 min) and the solvent was decanted. The extraction was repeated three times. Extracts from the three sequential extractions were reduced in volume by rotary evaporation (maximum 20 mbar) at 60 °C until dryness, spiked with secondary standard and evaporated again. Samples were reconstituted in 1 ml of aqueous mobile phase. Mussels were extracted in a similar manner except that the whole mussel tissue (7 g-14 g wet wt) was minced with stainless steel scissors and the tissue was placed into the centrifuge tube.

Polar organic chemical integrative samplers were extracted according to manufacturer's guidelines. Briefly, samplers were rinsed with ultrapure water to remove any material adhering to the surface. The disk was disassembled and the sorbent was extracted with 120 ml of

methanol. Methanol was filtered using UniPrep (Whatman), reduced under nitrogen, spiked with secondary standard and ultimately reconstituted in 1ml aqueous mobile phase. Samples were transferred to autosampler vials for analysis.

All analyses were performed with an Agilent 1100 liquid chromatograph coupled to an API 4000 triple-quadrupole mass spectrometer equipped with an electrospray interface operated in positive ionization mode (Applied Biosystems). Analytes were individually infused to determine the tandem mass spectrometer ion transitions and compound dependent parameters. Multiple daughter ions were monitored (multiple reaction monitoring) for confirmation purposes but quantification was based on a single transition. Additional mass spectrometer parameters and the description of quality assurance protocols used in the analyses are provided in detail in the Supplemental Data. A rigorous quality assurance protocol included the measurement of solvent blanks, procedural blanks, replicate analyses, matrix spikes, and primary internal standard recovery was followed during all analyses. Sediment and tissue analyte concentrations are reported on a wet weight basis. For mussel and sediment analysis, matrix spike recoveries ranged from 51 to 94 % and primary internal standard recovery averaged 70%. For water analysis, matrix spike recoveries were 80 to 100% and primary internal standard recovery averaged 85%. Laboratory replicate relative standard deviations were <15%. Limit of detection for fluoxetine ranged from 0.05 ng/g in sediment and mussel tissue to 1.5 ng per POCIS sampler. There were no detections in procedural and solvent blanks. The data were not corrected for recovery. Additional details regarding quality assurance/quality control measures are provided in the Supplemental Data.

Mussel toxicity testing

To assess the effects of fluoxetine on reproductive parameters with adult mussels in the lab, we collected mussels from two of the major Unionid tribes, Amblemini and Lampsilini. Representatives of the Amblemini tribe, *Elliptio complanata,* (length 55 mm-77 mm) were collected from the Eno River near Hillsborough and Little Creek near Wilson (NC, USA), in July 2004, June 2005, April 2006, and July 2006. Lampsiline species included *Lampsilis cardium* and *L. fasciola*. Adult female *Lampsilis cardium* (length 93 mm-123 mm) displaying mantle flap lures were collected from the Upper Mississippi River near La Crosse (WI, USA), in September 2005. Adult female *Lampsilis fasciola* (length 45 mm-82 mm) displaying mantle flap lures were collected from the Little Tennessee River near Franklin (NC, USA), in May 2006. With the possible exception of the Mississippi River, all mussels were obtained from relatively uncontaminated and rural areas. All mussels used in the present study were transported or shipped to the Aquatic Toxicology Lab at North Carolina State University using methods of Cope et al. [24]. Upon arrival at the laboratory, all mussels were acclimated to reconstituted soft water [25] over 24 h and maintained at 18 to 20°C for at least 24 h prior to beginning any experiments to ensure that spawning or release of glochidia during experiments was not a result of handling or transport stress. Approximately 21% (25/120) of female mussels returned to the lab released glochidia (>90% viable) during acclimation. Only female mussels that had not released glochidia during acclimation were used for toxicity tests.

Fluoxetine hydrochloride salt (Sigma-Aldrich; 98% purity) and serotonin creatinine sulfate (Acros Organics; 99% purity) were purchased from VWR International (West Chester). Working solutions of fluoxetine (8 mg/ml and 0.008 mg/ml) and serotonin (20 mg/ml) were prepared in deionized water. All test concentrations were calculated based on the concentration of free fluoxetine, as opposed to the salt form in which it was purchased. Test containers were

pre-conditioned with appropriate test solutions for 24 h before experiments began. Test solutions were prepared in reconstituted soft water [26] for all experiments. Temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness were measured according to standard methods [26] in one replicate of each treatment at least at the beginning and end of each test. A calibrated multiprobe (YSI Model 556 MPS, Yellow Springs Instruments) was used to measure pH, dissolved oxygen, conductivity and temperature. Alkalinity was determined by titration with 0.02 N H_2SO_4 to pH 4.5, and hardness by titration with 0.01 M ethylenediaminetetraacetic acid.

We assessed glochidia release by brooding female *E. complanata* following exposure to fluoxetine in three trials (July 2004, June 2005, and July 2006), each with six treatment concentrations (0, 0.3 µg/L, 3.0 µg/L, 30 µg/L, 300 µg/L, or 3000 µg/L), and 3 to 6 mussels per treatment for 96 h. We chose this range for the lab exposures because the lowest test concentrations were similar to the highest fluoxetine concentrations measured in the effluent (~0.12 µg/L) and because the lab experiments were all short-term (< 96 h) exposures. A serotonin treatment (40 mg/L) was included as a positive control [13,14,16]. The mussels were placed in 3.75-L glass aquaria (1 mussel/aquarium) containing 2 L of gently aerated reconstituted soft water andexamined at 24 h intervals for the duration of the exposure. Endpoints included time from initiation of exposure to parturition of >100 glochidia and viability of released glochidia, determined by response of a sub-sample (n = 50-100) of the released glochidia to a saturated solution of NaCl [27]. Composite water samples (10 ml from each replicate in a given treatment) were collected for analysis of fluoxetine at time 0 and at the time of first release of glochidia in a replicate. Substrate, either artificial (e.g., aquarium gravel) or natural sediment was not used in tests with *E. complanata*.

Effects of fluoxetine on mantle flap display behavior and glochidia parturition were evaluated in *L. cardium* in September 2005 and in *L. fasciola* in May 2006. Mussels were maintained in 3.75-L glass aquaria (1 mussel/aquarium) containing 2 L of gently aerated reconstituted soft water and 12 mm of artificial substrate (aquarium gravel) at 18 to 20°C. Mussels were exposed to one of five fluoxetine treatments (0, 0.3 µg/L, 3.0 µg/L, 30 µg/L, 300 µg/L, or 3000 µg/L) with 6 replicates per treatment for 96 h (n = 36 experimental units). All exposure water was renewed at 24-h intervals. Mussels were monitored for mantle flap display and the release of glochidia continuously for the first 6 h of the experiment and then observed at 2 h intervals during 8 h blocks over the remaining exposure duration. Mantle flap display behaviors were categorized as: stage 1- shell closed; stage 2- shell gaped but no mantle tissue exposed; stage 3- shell gaped and mantle tissue partially extended; stage 4- shell gaped and mantle tissue fully extended with fish lure visible; stage 5- shell gaped, fish lure fully extended, and marsupial gills extended beyond shell margin; stage 6- shell gaped, fish lure fully extended, marsupial gills fully extended, and fish lure pulsating (number of beats/min was quantified). In addition, time to release of glochidia (>100) was recorded and glochidia viability was determined. Composite water samples (200 ml from each of 6 replicates) were collected for fluoxetine analysis from each treatment at the time mussels were initially placed in fluoxetine treatments. Effects of fluoxetine on release of spermatozeugmata or "sperm spheres" [28] were evaluated in male *E. complanata* in April 2006. Because *E. complanata* is not a sexually dimorphic species, we initiated the experiment with 48 non-gravid adult mussels of unknown gender (length 52 mm-74 mm). Mussels were randomly assigned to one of three treatments: control, 300 µg fluoxetine/L, or 3000 µg fluoxetine/L. An experimental unit consisted of a 3.75-L glass aquarium with 1 mussel and 2 L of gently aerated reconstituted soft water [25]. Temperature

was maintained at 20 °C by partially submerging aquaria in a water bath (Living Stream®, Frigid Units). Water samples (10 ml) were collected from each aquarium at the start of the experiment (before addition of fluoxetine) and examined at 40x magnification under a dissecting microscope for the presence of spermatozeugmata or glochidia. No mussels had released gametes or glochidia by the start of the experiment. A release of spermatozeugmata was defined as >10 spermatozeugmata per 10-ml sample of exposure water and glochidia parturition was documented when > 100 glochidia were expelled. Water samples were collected and examined at 2 h intervals for the first 12 h of the experiment and from 24 to 36 h. A final 10-ml sample was examined at 48 h, after which mussels that had not released sperm or glochidia were exposed to serotonin (80 mg/L) for ≤ 8 h to stimulate release of spermatozeugmata or glochidia. Test solutions were renewed (100%) at 24 h. Test water exposure concentrations were extracted and quantified similar to that previously described for stream water samples.

Statistical analyses

The three glochidia release trials with *E. complanata* were treated as replicates in time and the mean percentage of mussels releasing glochidia in each treatment was determined (n = 21 experimental units). The JMP (SAS) statistical software was used to test for homogeneity of variances (Bartlett's Test) and differences in glochidia release between treatment groups and controls was evaluated with analysis of variance followed by Dunnett's Test ($\alpha = 0.05$). Display behavior (i.e., frequency of occurrence in each stage) was analyzed by multivariate analysis of variance followed by Dunnett's test to compare control and treatment means for each stage (α = 0.05, n = 36 experimental units). For simplicity, frequencies of occurrence results were combined into three categories: stages 1 and 2, stages 3 and 4, and stages 5 and 6. Preceding

analysis, frequencies of occurrence in each of the three categories were transformed (arcsine) to achieve homogeneity of variance.

RESULTS AND DISCUSSION

Environmental sampling

Fluoxetine (and other pharmaceuticals; Supplemental Data, Tables S1-5) was detected in each of the environmental samples (water, POCIS, sediment, mussels) taken in and downstream of the wastewater effluent channel. Mean measured concentrations of fluoxetine in water and POCIS samples were similar (<20% difference) within each site and were lowest at an upstream reference site (<limit of detection-2.4 ng/L), greatest in the effluent channel (104 ng/L-119 ng/L), and decreased at sites 50 m (10.0 ng/L-14.4 ng/L) and 100 m (5.1 ng/L-7.3 ng/L) downstream of the effluent channel in Crabtree Creek (**Fig. 1**a). The similarity in concentrations of fluoxetine in surface water samples and the POCIS devices at a given site indicates that there is a relatively consistent discharge of fluoxetine in the wastewater effluent and that fluoxetine is detected in measureable quantities for at least 100 m downstream in Crabtree Creek. The time-weighted average concentrations of fluoxetine provided by the POCIS devices were calculated based on the uptake rate for fluoxetine (0.196 L/d) determined under turbulent conditions for a 41 cm^2 POCIS device [29]. Based on our results, the POCIS sampling technology was a reliable tool for quantitatively assessing fluoxetine in surface waters and corroborates recent research by other investigators [30,31].

The mean measured concentrations of fluoxetine in sediment and mussel tissue showed the same patterns as for water and POCIS samples, with the effluent channel sediment (17.4 ng/g

wet wt) and mussels (79.1 ng/g wet wt) having the greatest concentrations (Fig. 1b). Longitudinally in Crabtree Creek, sediment fluoxetine concentrations ranged from 0.35 ng/g wet weight at the upstream reference site to 5.3 and 1.3 ng/g wet weight, respectively at sites 50 and 100 m downstream of the effluent channel. These results support previous studies that report the occurrence of fluoxetine in aquatic sediments and soils [32,33]. Caged mussels accumulated substantial quantities of fluoxetine in their tissues after only 14 d; however it is unknown if the fluoxetine had reached steady state in the mussels during this period. From a high of 79.1 ng/g wet weight measured in mussels from the effluent channel, tissue fluoxetine remained elevated at sites 50 m (18.0 ng/g wet wt) and 100 m (9.8 ng/g wet wt) downstream, compared to the upstream reference mussels, which had 0.3 ng fluoxetine/g wet weight.

Fluoxetine 14-d bioaccumulation factor estimates for mussels at the four sites ranged from 125 to 1347 and were calculated by dividing the tissue concentration by the water concentration (**Table 1**). These values are similar to reported predicted bioconcentration factors (BCF), based on octanol-water partitioning coefficient, under certain environmental conditions [34] but are greater than BCF estimates reported for this compound in Japanese medaka (*Oryzias latipes*; BCF = 74-80) by other investigators [35]. Bioconcentration factors do not account for uptake via dietary routes that may partially explain the differences between accumulation in mussels and fish from these studies. Additionally, we did not examine accumulation or depuration kinetics and the mussel fluoxetine concentrations may not have reached steady state within this period. Pharmacokinetics of fluoxetine in mussels remains an important area of research.

Mean mussel concentrations of fluoxetine were highly correlated with those measured in water ($r^2 = 0.986$, $p = 0.007$) and POCIS ($r^2 = 0.985$, $p = 0.008$) samples at the sites. These

strong relations indicate a rapid uptake of fluoxetine via waterborne exposure across the gills of mussels—presumably through suspension-feeding and respiratory activities. However, uptake and exposure to fluoxetine via sediments may have also occurred through their deposit feeding activity [19] and is of concern for potential long-term, chronic exposures of mussels residing in streams receiving treated wastewater. Moreover, the range of fluoxetine concentrations measured in mussels from the effluent channel and the downstream sites (9.8 ng/g-79.1 ng/g wet wt) were greater than those measured from a variety of fish species (range 0.1 ng/g-1.6 ng/g wet wt) in waters impacted by municipal effluent [5, 36], underscoring the concern for potential adverse effects of this, and other pharmaceuticals, on this already imperiled group of fauna. Measurements of fluoxetine were performed in mussels that were not depurated; therefore, body burdens may have been influenced by fluoxetine in the digestive tract. Future studies of unionid mussels and fluoxetine should also include the analysis of norfluoxetine, a primary metabolite of fluoxetine, which has been shown to induce spawning and parturition in other bivalves [37] and to accumulate in fish [5,36].

Mussel toxicity testing

A significantly greater percentage of mussels in the 300 µg/L ($p = 0.012$) and 3000 µg/L ($p < 0.001$) fluoxetine treatments released nonviable (immature) glochidia compared to controls (**Fig. 2**). Mussels in the 0.3 µg/L fluoxetine treatment also released nonviable glochidia; however, this was not significantly different from controls ($p = 0.999$). One control mussel in the June 2005 trial released nonviable glochidia but no control mussels released nonviable glochidia in the other two trials. Some mussels in each treatment released viable glochidia, but there was not a significant difference ($p = 0.818$) in the percentage of mussels in each treatment that

released viable glochidia (Fig. 2). Glochidia release by mussels exposed to serotonin, the positive control, was similar to the release by mussels exposed to the highest fluoxetine concentration (Fig. 2). Mussels exposed to 300 µg/L and 3000 µg/L of fluoxetine and serotonin had marked increase in foot volume (discussed below), many to the point that they were unable to close their shell.

Fluoxetine substantially altered mantle flap lure display behavior in *L. cardium* and *L. fasciola* in a similar pattern. In *L. fasciola*, for example, mussels exposed to 300 µg/L (p = 0.0075) and 3000 µg/L ($p < 0.001$) of fluoxetine were more frequently observed in the most advanced stages of display (stages 5 and 6) compared to control mussels. Concomitantly, mussels in the 300 µg/L ($p = 0.034$) and 3000 µg/L ($p = 0.001$) fluoxetine treatments were less frequently observed in less advanced display stages (stages 1 and 2) compared to control mussels (**Fig. 3**). The trend toward more advanced stages of display behavior was more pronounced in mussels exposed to 3000 µg/L of fluoxetine compared to the 300 µg/L treatment group, indicating a concentration-response relationship, which may be mediated through serotonergic neurons [38]. Additionally, variability of observation frequency in the various stages among replicates also decreased in the mussels exposed to 3000 µg/L (Fig. 3). The ecological significance of altered mantle lure display behavior is not fully understood and requires further evaluation; however, mantle lure displays are intended to entice fish attacks so that glochidia can be expelled on to the gills of the fish and unnecessary tissue damage from fish strikes may result in damaged lures that are less effective at attracting host fish during the reproductive period when the mussels are brooding glochidia.

The results of the experiment with *L. cardium* were consistent with those of *L. fasciola* in terms of a concentration-related response in mantle lure display behavior, except that only

mussels in the 3000 µg/L fluoxetine treatment were more frequently observed ($p = 0.0001$) in the most advanced stages of display (stages 5 and 6) compared to control mussels (data not shown). Parturition of glochidia occurred during the mantle lure display experiments with *L. cardium* and *L. fasciola*, as in experiments with *E. complanata*. However, in both of these tests, only mussels in the 3000 µg/L fluoxetine treatment released a significantly ($p < 0.0001$) greater percentage of glochidia compared to controls. All of the glochidia released from mussels during these two experiments were viable glochidia, unlike in the tests with *E. complanata*, in which viable and nonviable glochidia were released. In these tests, the number of glochidia released by mussels exposed to serotonin, the positive control, was again similar to those released by mussels exposed to the highest fluoxetine concentration. Also, as observed in the experiments with *E. complanata*, the foot volume of *L. fasciola* and *L. cardium* was substantially increased (discussed below) in mussels exposed to the two highest fluoxetine concentrations.

Fluoxetine induced release of spermatozeugmata in 7 of 16 (44%) *E. complanata* of unknown gender in the 3000 µg/L treatment, whereas 1 of 16 (6%) mussels in each of the control and 300 µg/L fluoxetine treatments released spermatozeugmata. Release of spermatozeugmata by mussels in the 3000 µg/L fluoxetine treatment was significantly different ($p = 0.0056$) from other treatments. In addition, 5 of the 16 (31%) mussels in the 300 µg/L fluoxetine treatment and 4 of 16 (25%) in the 3000 µg/L fluoxetine treatment released glochidia, whereas 2 of 16 (13%) released glochidia in the control treatment. Of the 12 remaining mussels that had been exposed to fluoxetine but had not released spermatozeugmata or glochidia within 48 h, 2 released nonviable glochidia within 2 h of exposure to serotonin (80 mg/L). The 10 remaining mussels did not release glochidia or gametes within 24 h of exposure to serotonin.

Our results demonstrate that fluoxetine has the potential to disrupt reproduction in native mussel species by inducing release of spermatozeugmata and glochidia as well as altering mantle flap display behavior. The ecological effects of an ill-timed release of glochidia or gametes caused by fluoxetine exposure could be potentially devastating to localized mussel populations. Likewise, the inability of a female mussel to attract a suitable host fish (for glochidia infestation) through altered or ill-timed mantle flap (fish lure) display behavior could also result in reproductive failure. The lowest-observed-effect concentration (LOEC) for fluoxetine in the present study was 300 μg/L and the no-observed-effect concentration (NOEC) was 30 μg/L, thus the true threshold for effects lies somewhere between 30 μg/L and 300 μg/L. The LOEC of 300 μg/L fluoxetine for adult mussel reproductive effects in the present study is similar to effects concentrations reported for some other invertebrate species that have been tested [38,39]. The LOEC was substantially greater (several orders of magnitude) than the concentrations measured in samples of water from the treated effluent in the present study (0.119 μg/L) and in samples from other locations (maximum 0.142 μg/L) [3]; however, our test durations for all experiments were relatively short (≤ 96 h) relative to the lifespan of these animals. Fluoxetine has been demonstrated to cause adverse effects in full lifecycle tests with short-lived aquatic invertebrates (*Daphnia magna* and the snail *Potamopyrgus antipodarum*) at concentrations as low as 10 μg/L [39]. It is currently unclear how the results of our acute fluoxetine toxicity tests relate to results of chronic exposures in these long lived mussels but based on the findings of Péry et al. [40] who tested chronic effects of fluoxetine with another mollusk, it is reasonable to hypothesize that LOECs for chronic studies with mussels would be similar to those reported for another mollusk. An LOEC of 10 μg/L is still at least an order of magnitude greater than the highest concentrations measured in the environment in the present study but the possibility of the

presence of other SSRIs with similar modes of action and their additive or synergistic effects should not be ignored. Moreover, the effects of fluoxetine on the sensitive early life stages of mussels (glochidia and juveniles) have not been assessed and little is known about the effects of chronic, low-level fluoxetine (and other SSRI) exposure on mussel reproduction in actual stream settings.

Fluoxetine and other SSRI drugs have been used previously to induce spawning and parturition in mussels. Cunha and Machado [13] used fluoxetine and fluvoxamine, another SSRI drug, to control timing and intensity of glochidia release in *Anodonta cygnea,* for aquaculture purposes. Significant numbers of viable glochidia were released by gravid mussels exposed to 309 and 3090 µg/L of fluoxetine during a 24-h exposure period in their study. *Elliptio complanata* in the present study appeared to be similarly sensitive to fluoxetine. Additionally, consistent with reports by Cunha and Machado [13] for *A. cygnea*, we observed a strong increase in volume of the foot in *E. complanata* during early stages of exposure to fluoxetine and serotonin. Cunha and Machado [13] attributed the increase in foot size to relaxation of foot muscles and the resulting favorable conditions for uptake and storage of water.

To our knowledge, this is the first report of reproductive effects with male unionid mussels exposed to an SSRI drug. Male *E. complanata* exposed to 3000 µg fluoxetine/L released spermatozeugmata (aggregates of hundreds to thousands of spermatozoa) but those exposed to the next lowest concentration, 300 µg/L, did not. Fong [39] reported induction of spawning in zebra mussels at 155 µg/L fluoxetine for males and 1545 µg/L for females. The effects of fluoxetine on viability of spermatozeugmata or individual spermatozoa after release from the sperm sphere have not been elucidated.

Consistent with other studies of mollusks in which fluoxetine stimulated reproductive processes [39,41], fluoxetine exposure resulted in a concentration-dependent stimulation of mantle lure display behaviors by *L. fasciola* and *L. cardium*. It is currently unclear if such behavioral effects would preclude successful attraction of a suitable host fish for glochidia infestation. Additionally, it is not known if fluoxetine (or other SSRIs) would induce display behaviors in female mussels that are not gravid (i.e., out of season), or prematurely induce displays in those that contain immature or nonviable glochidia. Additional research is required to elucidate the effects of fluoxetine on other ecologically-relevant behaviors such as burrowing and feeding. Because SSRIs are most commonly associated with municipal wastewater effluents, they generally exist as mixtures rather than individual chemicals in surface waters. It would be prudent to evaluate the potential for surface waters to disrupt neuroendocrine pathways based on total SSRI concentration and activity rather than focus on individual chemicals because the combined total SSRI activity may better estimate effects on critical biological endpoints.

Acknowledgement−The Water Resources Research Institute of The University of North Carolina and U.S. Geological Survey provided funding for this project. We thank P. Lazaro, S. Prochazka, S. Mosher, T. Pandolfo, and E. Tracy for technical assistance in the field and laboratory. Consuelo Arellano in the Department of Statistics at North Carolina State University provided assistance with statistical analyses. We thank F. and C. Barnhart for their thorough and helpful review of this manuscript. Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and

Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

REFERENCES

1. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect* 107:907-938.
2. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol* 36:1202-1211.
3. Metcalfe CD, Miao XS, Koenig BG, Struger J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the Lower Great Lakes, Canada. *Environ Toxicol Chem* 22:2881-2889.
4. Vieno NM, Harkki H, Tuhkanen T, Kronberg L. 2007. Occurrence of pharmaceuticals in river water and their elimination in a pilot-scale drinking water treatment plant. *Environ Sci Technol* 41:5077-5084.
5. Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ Toxicol Chem* 24:464-469.
6. Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378-384.
7. Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vitro responses in trout and roach. *Environ Sci Technol* 32:1559-1565.
8. Ternes TA. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res* 32:3245-3260.

9. Fong PP, Duncan J, Ram JL. 1994. Inhibition and sex specific induction of spawning by serotonergic ligands in the zebra mussel *Dreissena polymorpha* (Pallas). *Experientia* 50: 506-509.
10. Hirai S, Kishimoto T, Kadam AL, Kanatani H, Koide SS. 1988. Induction of spawning and oocyte maturation by 5-hydroxytryptamine in the surf clam. *J Exp Zool* 245:318-321.
11. Ram, JL, Crawford GW, Walker JU, Mojares JJ, Patel N, Fong PP, Kyozuka K. 1993. Spawning in the zebra mussel (*Dreissena polymorpha*): activation by internal or external application of serotonin. *J Exp Zool* 265:587-598.
12. Fong PP, Wade S, Rostafin M. 1996. Characterization of serotonin receptor mediating parturition in fingernail clams *Sphaerium* (Musculium) spp. from eastern North America. *J Exp Zool* 275:326-330.
13. Cunha EM, Machado J. 2001. Parturition in *Anodonta cygnea* induced by selective serotonin reuptake inhibitors (SSRIs). *Can J Zool* 79:95-100.
14. Gibbons MC, Castagna M. 1984. Serotonin as an inducer of spawning in six bivalve species. *Aquaculture* 40:189-191.
15. Ram JL, Fong PP, Croll RP, Nichols SJ, Wall D. 1992. The zebra mussel (*Dreissena polymorpha*), a new pest in North America: reproductive mechanisms as possible targets of control strategies. *Invertbr Reprod Develop* 22:77-86.
16. Bogan AE. 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): A search for causes. *Am Zool* 33:599-609.
17. Williams JD, Warren ML, Cummings KS, Harris JL, Neves RJ. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18:6-22.

18. Strayer DL, Downing JA, Haag WR, King TL, Layzer JB, Newton TJ, Nichols SJ. 2004. Changing perspectives on pearly mussels, North America's most imperiled animals. *BioScience* 54:429-439.

19. Cope WG, Bringolf RB, Buchwalter DB, Newton TJ, Ingersoll CG, Wang N, Augspurger T, Dwyer FJ, Barnhart MC, Neves RJ, Hammer E. 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. *J North Am Benthol Soc* 27:451-462.

20. Barnhart MC, Haag WR, Roston WN. 2008. Adaptations to host infection and larval parasitism in the Unionoida. *J North Am Benthol Soc* 27:370-394.

21. Haag WR, Butler RS, Hartfield PD. 1995. An extraordinary reproductive strategy in freshwater bivalves: prey mimicry to facilitate larval dispersion. *Freshw Biol* 34: 471-476.

22. Haag WR, Warren ML. 1999. Mantle displays of freshwater mussels elicit attacks from fish. *Freshw Biol* 42:35-40.

23. U.S. Geological Survey. 1998. Low-flow characteristics and discharge profiles for selected streams in the Neuse River Basin, North Carolina. Water-Resources Investigations Report 98-4135. Washington, DC.

24. Cope WG, Hove MC, Waller DL, Hornbach DJ, Bartsch MR, Cunningham LA, Dunn HL, Kapuscinski AR. 2003. Evaluation of relocation of unionid mussels to in situ refugia. *J Mollus Stud* 69:27-34.

25. American Society for Testing and Materials. 1993. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. E729-88a. West Conshohocken, PA.

26. Greenberg AE. 1995. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.

27. American Society for Testing and Materials. 2007. *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels.* E2455-07. West Conshohocken.

28. Ishibashi R, Komaru A, Kondo T. 2000. Sperm sphere in Unionid mussels (Bivalvia: Unionidae). *Zool Sci* 17:947-950.

29. Alvarez DA, Petty JD, Huckins JN, Jones-Lepp TL, Getting DT, Goddard JP, Manahan SE. 2004. Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments. *Environ Toxicol Chem* 23:1640-1648.

30. Alvarez DA, Stackelberg PE, Petty JD, Huckins JN, Furlong ET, Zaugg SD, Meyer MT. 2005. Comparison of a novel passive sampler to standard water-column sampling for organic contaminants associated with wastewater effluents entering a New Jersey stream. *Chemosphere* 61:610-622.

31. MacLeod SL, McClure EL, Wong CS. 2007. Laboratory calibration and field deployment of the polar organic chemical integrative sampler for pharmaceuticals and personal care products in wastewater and surface water. *Environ Toxicol Chem* 26:2517-2529.

32. Kwon J-W, Armbrust KL. 2006. Laboratory persistence and fate of fluoxetine in aquatic environments. *Environ Toxicol Chem* 25:2561-2568.

33. Kinney CA, Furlong ET, Werner SL, Cahill JD. 2006. Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environ Toxicol Chem* 25:317-326.

34. Paterson G, Metcalfe CD. 2008. Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). *Chemosphere* 74:125-130.

35. Brooks, B, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, LaPoint TW. 2003. Aquatic ecotoxicology of fluoxetine. *Toxicol Lett* 142:169-183.

36. Chu S, Metcalfe CD. 2007. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 1163:112-118.

37. Fong PP, Molnar N. 2008. Norfluoxetine induces spawning and parturition in estuarine and freshwater bivalves. *Bull Environ Contam Toxicol* 81:535-538.

38. Ram JL, Moore D, Putchakayala S, Paredes AA, Ma D, Croll RP. 1999. Serotonergic responses of the siphons and adjacent mantle tissue of the zebra mussel, *Dreissena polymorpha*. *Comp Biochem Physiol C* 124:211-220.

39. Fong PP. 1998. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. *Biol Bull* 194:143-149.

40. Péry ARR, Gust M, Vollat B, Mons R, Ramil M, Fink G, Ternes T, Garric J. 2008. Fluoxetine effects assessment on the life cycle of aquatic invertebrates. *Chemosphere* 73:300-304.

41. Uhler GC, Huminski PT, Les FT, Fong PP. 2000. Cilia-driven rotational behavior in gastropod *(Physa elliptica*) embryos induced by serotonin and putative serotonin reuptake inhibitors (SSRIs). *J Exp Zool* 286:414-421.

Figure Legends

Fig. 1. Mean measured concentrations of fluoxetine in samples of (**A**) water, polar organic chemical integrative samplers (POCIS), (**B**) sediment, and caged freshwater mussels *Elliptio complanata* at sites upstream, in, and downstream (50 m and 100 m) of a municipal wastewater treatment facility effluent (Crabtree Creek) near the Town of Cary (NC, USA). Error bars indicate one standard deviation.

Fig. 2. Mean percent of gravid female *Ellipto complanata* (n = 14) per treatment that released glochidia during a 48-h exposure to fluoxetine (0 - 3000 µg/L) or serotonin (40 mg/L). Error bars indicate one standard deviation. Asterisks indicate a significant difference compared to control (Dunnett's Test): * $p \leq 0.05$, *** $p \leq 0.0001$.

Fig. 3. Summary of mantle flap display behavior for gravid female *Lampsilis fasciola* exposed to fluoxetine (0 - 3000 µg/L) for 96 h. Mean percent of observations (n = 16 total observations) per display stage (n = 6 replicates). Stages 1 and 2 were no visible mantle flap, stages 3 and 4 were partial displays, and stages 5 and 6 were full displays. Error bars indicate one standard deviation. Asterisks indicate a significant difference compared to control (Dunnett's Test): * $p \leq 0.05$, *** $p \leq 0.001$.

Table 1. Partitioning characteristics of fluoxetine in environmental samples collected near a municipal wastewater effluent discharge in Cary (NC, USA).

Site	Mussel 14-d BAF[a]	K_d[b]
Upstream reference	125	146
Effluent	665	146
50 m downstream	1250	368
100 m downstream	1347	178

[a] Freshwater mussel (*Elliptio complanata*) bioaccumulation factor = [tissue (ng/g)]/[water (ng/ml)]

[b] Sediment-water partitioning coefficient =[sediment (ng/g)]/[water (ng/ml)]

Figures

Fig 1.

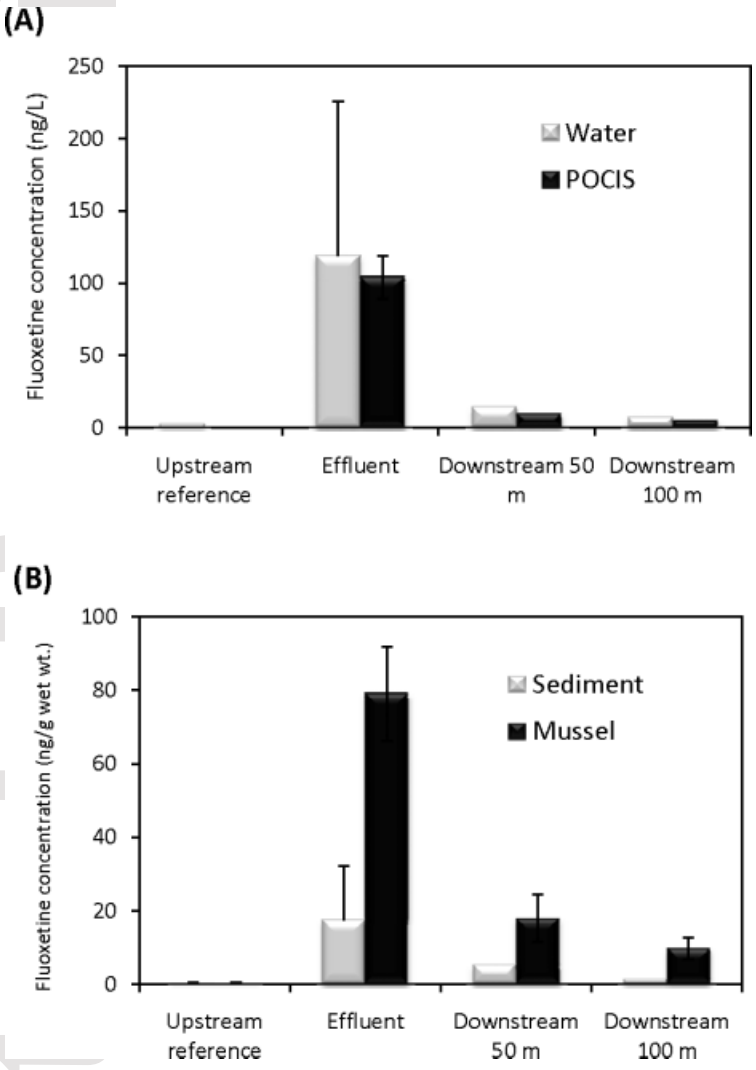

Fig 2.

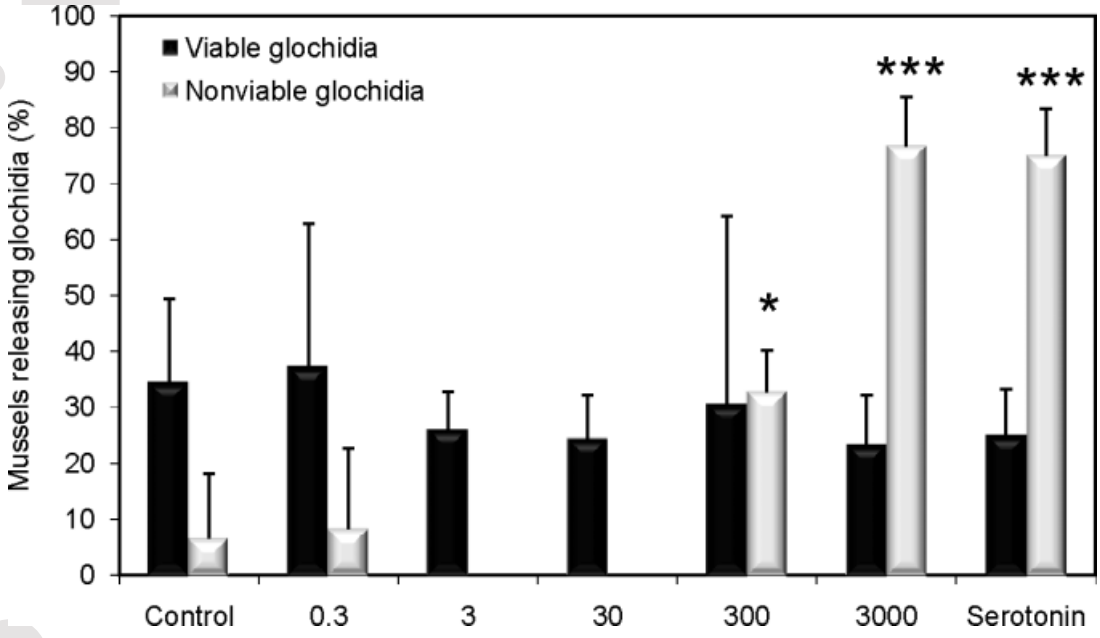

Fig 3.